

History of the discoveries of the first human retroviruses: HTLV-1 and HTLV-2

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HTLV-1 was discovered in the US in 1979, and published in 1980. This was rapidly followed by four additional reports in early 1981 describing additional isolates, characterization of some of the HTLV-1 proteins, serological assays for specific antibodies indicative of HTLV-1 infection, and evidence for integrated DNA proviruses in infected cells. None of this early work was dependent upon or influenced by the subclassification of some T-cell malignancies as ATL (in Japan). Instead, I was stimulated by prior work from many investigators in the US and Europe on retroviruses which caused leukemia in animals and our discoveries were made possible by our technical approaches developed in the 1970s involving especially sensitive assays for RT as a surrogate marker for a retrovirus and our discovery of IL-2 which made it possible to culture human T cells. However, following our reports the same virus was isolated in Japan, and both groups provided evidence that HTLV-1 caused ATL, a subclassification of T-cell malignancies first recognized in Japan.

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The years of preparation that led to the discoveries of human retroviruses

Discovery of lymphokines and early hints of human retroviruses

My interests have focused on the biology of human blood cells and a comparison of normal and leukemic blood cells (Gallo *et al.*, 1967; Gallo and Breitman, 1968a, b; Gallo and Pestka, 1970; Smith *et al.*, 1975). Initially, I emphasized acute lymphocytic leukemias (ALL) because these were common leukemias and gram quantities of these cells were available. Moreover, they were the only leukemia's for which normal control cells were available, namely, normal human lymphoblasts. The plant lectin, phytohemagglutinin (PHA), was known to induce human lymphocytes to become activated and go through a mitotic cycle. Their growth,

however, was severely limited. Generally, cells only survived for about 1 week, and their proliferation was limited to one cycle. These normal lymphoblasts looked like ALL cells, but these were days before most of us could know of the complexity of subtypes of lymphocytes. Functional discriminatory assays were barely available and monoclonal antibodies with their capacity to provide surface markers were yet to come. We learned how to purify lymphocytes from columns packed with nylon; myeloid cells would adhere, but lymphocytes passed through. Herb Cooper of NIH generously provided this technique to me. During this period (1968–1970) I became very impressed by the studies of Leo Sachs in Israel and of Don Metcalf in Australia who were showing that, like some lymphocytes, myeloid cells could also be grown in the laboratory but not in liquid culture. Instead, they used the technique previously applied to virus-transformed cells of cell growth on a methylcellulose solid surface in the form of cell colonies. However, here too growth was very limited as was the amount of cells obtained, precluding many types of biochemical, molecular biological, and virological experiments. Nonetheless, from this system, Sachs and his colleagues and Metcalf and co-workers made seminal discoveries, including a growth/differentiation factor, granulocyte macrophage colony-stimulating factor (GM-CSF), which was specific for the myeloid lineage, but it was thought that the main production of GM-CSF would be from myeloid cells, that is, a feedback regulation – granulopoietic progenitors proliferated and formed 'dead end' granulocytes, which presumably would produce their own granulopoietic factor (Metcalf, 1979; Lotem and Sachs, 1986).

Meanwhile, while comparing ALL cells to normal lymphocytes, I decided to test the conditioned medium of the PHA-stimulated normal cells for growth factors. With the late Alan Wu and Joan Prival, we reported finding that lymphocytes (T cells) made GM-CSF (Prival *et al.*, 1974). This would be the start of my long involvement with 'conditioned medium' from PHA-stimulated lymphocytes. Dane Boggs, F Ruscelli, and co-workers in Pittsburgh had described the same phenomena at almost exactly the same time. These papers were likely among the first to describe lymphokines (lymphocyte-derived cytokines).

In the early 1970s, I also began to study animal retroviruses because in several animals these kinds of viruses caused leukemia's. Whether human retroviruses

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existed or not, a study of animal retroviruses especially focused on learning their leukemogenic mechanisms, might provide insights into the mechanisms involved in human leukemia's. My co-workers and I also decided to search for human retroviruses, an unpopular goal at this time considering the decades of attempts and failures. I was mainly encouraged in this effort by discussions with William Jarrett, the Scottish veterinarian who discovered feline leukemia virus, and by the work of the late Howard Temin. Temin, of course, had predicted that retroviruses of animals replicated by having their RNA genome transcribed into a DNA form, which would then integrate into the DNA of the target cell. He referred to this integrated form as provirus, the name given to his theory. In 1970 Temin and his colleague Mizutani, and separately, David Baltimore, gave credence to the theory with their discovery of the DNA polymerase carried by all retroviruses, reverse transcriptase (RT) (Baltimore, 1970; Temin and Mizutani, 1970). For me it also meant a possible convenient, inexpensive, and extremely sensitive assay for a retrovirus. (This would be one of two technologies that would be key for later discoveries of all human retroviruses.)

RT forms in virions only upon budding from the cell. Consequently, finding this enzyme in media of cultured cells implied release of retrovirus particles, and finding RT from extracts of cells implied the presence of a cell associated virus particles, as for example, virions associated with the cell surface membrane. We found rare cases of leukemia that scored positive in RT assays. The problem, however, was that the normal cells have several DNA polymerases, and some have similarities to RT. We needed to develop the assay not only as a very sensitive one but also one that would distinguish RT from all of the then known cellular DNA polymerases (alpha, beta, and gamma). This became a major objective (Bobrow *et al.*, 1972; Robert *et al.*, 1972; Reitz, Jr *et al.*, 1974; Smith *et al.*, 1975; Robert-Guroff and Gallo, 1977).

Ultimately, we did find a few cases of lymphocytic leukemia's in adults with RT showing all the characteristics of RT from a retrovirus (we had by then purified and characterized RT from many different animal retroviruses). We published on the one best characterized in *Nature New Biology* in 1972 (Sarngadharan *et al.*, 1972). I believed then and I believe now that this was indeed a 'footprint' of a human retrovirus, but we failed to isolate virus from this patient, that is, we failed to perpetuate virus in cell culture.

The common approach to virus isolation was by using cell lines. Cell culture technology had become widely available by the 1960s, and many cell lines from different species were available. The primary cells suspected of containing virus are co-cultured with a variety of such cell lines with the hope that the virus will 'take' in one or more. However, by this period, there was intense antagonism to research directed toward the finding of human tumor viruses and especially of retroviruses. The NCI had created the heavily funded Virus Cancer Program, which was under attack for failing to find clear evidence of tumor viruses. More-

over, by the mid-1970s there had been decades of failure to find human retroviruses, and many false starts that involved cell lines, including one by me. The usual problem was a cross contamination with an animal retrovirus. For this reason I became convinced that we had to find ways to grow primary blood cells, but not with the systems of Sachs and Metcalf. These methylcellulose colonies of leukocytes provided too few cells and growth of these cells were limited in number and in time.

We set about to search for growth factors, specifically proteins that would promote proliferation of primary blood cells. Our approach was to culture different types of cells from different tissue sources (including human embryos) for several days, collect the media (conditioned media or CM), and add it to leukocytes from normal human cord blood, samples of human bone marrow, or leukemic cells to see if we could get these cells to grow with the putative growth factor from a particular CM. Of course, CM from PHA-stimulated lymphocytes was one of the cell sources that we tested, and with my postdoctoral fellow D Morgan and with F Ruscetti, we discovered a T-cell growth factor in this CM. We reported these results in 1976–1977 (Morgan *et al.*, 1976; Ruscetti *et al.*, 1977) and they were to be the first reports of what we termed a T-cell mitogenic factor, later called TCGF, and finally interleukin-2 (IL-2). The purification was performed later (Mier and Gallo, 1982). IL-2 was among the first well-defined cytokines. The combination of IL-2 growth of T cells with sensitive RT assays would be (and still is) the key to the discoveries of human retroviruses in T-cell leukemias and AIDS.

Why the consensus against the possible existence of human retroviruses?

At the same time of the above observations pressure mounted against attempts to find human retroviruses. It was not only the history of failure but also scientific arguments such as: (1) the little evidence for leukemia viruses in primates; (2) the knowledge that when retroviruses were found in animals they were not difficult to find. Extensive viremia preceded disease; therefore, if they infected humans, they would be easy to find and would have been discovered much earlier. (3) Human sera in the presence of complement lysed animal retroviruses, thereby providing a rational mechanism for the conclusion that humans were protected against infections by retroviruses.

I thought otherwise. In the early 1970s Kawakami *et al.* (1972) had discovered gibbon ape leukemia virus (GALV), and linked it to chronic myeloid leukemia in that species. Later in the 1970s, we discovered a variant of that virus which caused T-cell leukemia (Gallo *et al.*, 1978). Bovine leukemia virus (BLV) was discovered (Ferrer *et al.*, 1975; Kettmann *et al.*, 1975), and it was noted that BLV replicated at very low levels, thus putting to rest the notion of 'extensive viremia always precedes animal retrovirus-induced leukemia's'. As for human sera-lysing retroviruses, unfortunately those studies were limited to tests of retroviruses from

nonhuman primates. Later, we would learn that many primate retroviruses, including the retroviruses of humans, are not susceptible to lysis by human serum/complement.

A focus on T-cell malignancies

Our ultimate focus on T-cell leukemia's was dictated by several factors. First, most animal leukemias caused by retroviruses are lymphocytic leukemias and of these T-cell leukemias predominate. Second, the first and to this date only leukemia of nonhuman primates is caused by a retrovirus, namely the leukemia of gibbon apes caused by GALV (Kawakami *et al.*, 1972; Gallo *et al.*, 1978), and a particular strain of this virus which we isolated caused T-cell leukemia (Gallo *et al.*, 1978). Third, fortune dictated that we would end up focusing on human T-cell malignancies because on our discovery of IL-2 which allowed us to grow significant numbers of such cells in liquid culture through many cell cycles. This was possible with normal T cells and some T-cell malignancies (not all T-cell leukemias or lymphomas respond to IL-2).

I was also influenced to continue a pursuit of human retroviruses by a documented interspecies transmission of a GALV from a pet old world Gibbon ape to a new world Woolly monkey, giving rise to the virus from the Woolly monkey now known as simian sarcoma virus (Wong-Staal *et al.*, 1981). It was well known that retroviruses could move from one species to another, but in all cases these were very ancient events only discovered by analyses of cellular DNA of many animals. But here was an example we witnessed suggesting such events are not rare and I felt humans could not be excluded, and indeed later we would learn that the first human retrovirus discovered (HTLV-1) has close relatives among many old world primates and may have arisen from an ancient transmission from monkey to man. A more relevant example, of course, is HIV. There is much evidence that it came into humans as a much more recent infection from African primates.

Discoveries of HTLV-1 and HTLV-2

HTLV-1 was first found by my co-workers and myself in 1979 from an analysis of a T cell line established by J Minna and his co-workers, P Bunn and A Gazdar from a patient these clinicians called a cutaneous T-cell lymphoma. Alternatively, such patients were also called mycosis fungoides or Sezary T-cell leukemia depending upon clinical nuances. Although IL-2 was supplied by us for them to use in their initial culturing of these cells, the cells rapidly immortalized and the IL-2, therefore, no longer needed. An outstanding postdoctoral fellow, Bernard Poiesz, carried out RT assays on these cells with positive results, and we soon arranged for electron microscopic analysis of concentrated RT plus cultures and found retrovirus particles. I was well aware that much more had to be done before this work was acceptable. For instance, we had to (1) show that the

same virus could be isolated from primary tissue samples of the same patient by culturing primary T cells with IL-2; (2) demonstrate that the virus was novel, that is, not any of the known animal retroviruses; (3) show it could infect human T cells *in vitro*; (4) demonstrate specific antibodies to the virus in the serum of the patient; (5) demonstrate that proviral DNA could be found integrated in the DNA of the cells from which the virus was isolated; (6) provide evidence that this was not a one-time affair by showing serological evidence of specific antibodies not only in the patient but in some others as well. These results were successfully obtained and available by the time we submitted our first report in 1980 (Poiesz *et al.*, 1980), enabling us to follow quickly with several other reports (Kalyanaraman *et al.*, 1981; Poiesz *et al.*, 1981; Reitz, Jr *et al.*, 1981; Rho *et al.*, 1981; Robert-Guroff *et al.*, 1981, 1982), also including isolates from other patients (Poiesz *et al.*, 1981). These and all subsequent isolates of HTLV-1 in our laboratory were from primary cells cultured with IL-2. It soon became clear that HTLV-1 was specifically associated with adult T-cell malignancy (usually CD4+ cells) in which the patients frequently had cutaneous abnormalities and hypercalcemia, but clinicians in the United States had not at that time made any distinction of HTLV-1-associated T-cell malignancies from other neoplasm's, and as noted above collectively referred to these patients with others (nonHTLV associated) as cutaneous T-cell leukemia-lymphomas. However, a few years earlier Kiyoshi Takatsuki and his co-workers T Uchiyama, and Junji Yodoi defined clusters of leukemia in southwest Japan with special clinical features and cellular morphology, which when coupled with the geographic clustering, led him to propose in 1977 that this was a distinct form of leukemia. He named it adult T-cell leukemia (ATL) (Uchiyama *et al.*, 1977).

Further progress by our group in the understanding of HTLV-1 and its role in T-cell malignancies came from information provided by T Waldmann and T Uchiyama, (who had joined Waldmann). They brought to our attention the ATL cluster in Japan, so in the fall of 1980, I contacted two Japanese friends, the late Yohei Ito, then Chair of Microbiology at Kyoto University and Tad Aoki for more information and for sera from such patients to test for antibodies to HTLV. Aoki and Ito sent sera from such patients to me in 1980, and these sera scored positive for antibodies to HTLV-1. Based on these results Ito organized a small meeting at Lake Miwa outside of Kyoto in March 1981. It was attended by a few co-workers and myself from the US and Aoki, Ito, and several other Japanese scientists most notably Takatsuki, Y Hinuma, and T Miyoshi. My colleagues and I presented our results, including a description of several isolates of HTLV-1, characteristics of purified HTLV-1 p24 as well as p19 and reverse transcriptase proteins, evidence of integrated HTLV-1 provirus, and the positive serological results in US and Japanese ATL patients, which provided clear evidence for the linkage of HTLV-1 to certain T-cell malignancies. The meeting summary was published in Cancer Research in November 1981 (Gallo, 1981).

At the end of the meeting when we were summarizing and planning for this collaboration with the Japanese investigators, Dr Y Hinuma announced he too had a retrovirus. He presented EM pictures of virus particles from a cell line established by I Miyoshi. Hinuma called his isolate adult T-cell leukemia virus (ATLV), but argued against collaboration claiming it was not possible to provide human sera from Japan for 'cultural reasons'. In October 1981 Hinuma *et al.* (1981) published for the first time on their isolate of ATL. Miyoshi had established his cell line by co-cultivation of ATL cells and normal human T cells. These results of Miyoshi were the first indication of the transforming capability of HTLV-1 because the cell line that was immortalized was from the normal donor (Miyoshi *et al.*, 1981). Later, my colleague M Popovic made this a routine. We showed that HTLV-1 could routinely immortalize normal human T cells (Popovic *et al.*, 1983). After comparative analyses of isolates of ATL and HTLV were performed, we published with Japanese colleagues M Yoshida, T Miyoshi and Y Ito that HTLV-1 and ATL were the same virus. Consequently, we agreed that the virus name should be HTLV to recognize the priority of our virus work, and the disease would be referred to as ATL in recognition of the Japanese priority in distinguishing this malignancy as a specific identity which had been 'lumped' with other T-cell leukemia's/lymphomas in western countries and elsewhere as cutaneous T-cell lymphomas.

A second meeting of historical note was in London and chaired by the late hematologist Sir John Dacie and attended by Drs Daniel Catovsky, Robin Weiss, Mel Greaves, and William Jarrett among others from Great Britain and by my collaborator in epidemiological studies, Dr William Blattner, and myself. It was Catovsky who called for this meeting because he noted that we had found HTLV-1 mainly in African-Americans and black persons in the Caribbean and he had found an unusual frequency of adult T-cell malignancies in Caribbean immigrants to England. He recognized the similarities of their disease to Takatsuki's ATL. Thus, he postulated they were one and the same disease and HTLV-1 would be present in all. He was right. Promptly, Blattner accelerated his studies in the Caribbean and documented that HTLV-1 was endemic in some islands. He and Guy de Thé of France would then show that this result depended upon the particular tribes in Africa from which the individuals descended.

Experiences with HTLV would often be predictive for the future, and notably for the HIV epidemic. The modes of transmission as sexual contact, blood, and mother to child via breast feeding, the CD4T cell tropism, the relatedness to primate and ungulate retroviruses, and the presence of regulatory genes are

examples. Later in 1981, we isolated HTLV-2 from a leukemia described as 'a hairy cell T-cell leukemia' and reported on it in 1982 (Kalyanaraman *et al.*, 1982).

Legacy of discoveries of the HTLVs with emphasis on their role in the discovery of HIV

Of course the first and most obvious legacy of the HTLV's are their historical role as the first and only known infectious cause of a human leukemia, and the first clear-cut viral origin of a human cancer. Second, is the information on the molecular mechanism of a leukemia, which might give insights into the mechanisms of other malignancies the vast majority of which we have no known cause. A third contribution is practical, namely, the capacity of the HTLVs to immortalize CD4+ and CD8+ T cells. For example, such cell lines have been used as sources of cytokines. Fourth, the observations with HTLV and technological developments leading to their discoveries were important for the discovery of HIV and its identification as the causative agent of AIDS. This includes: (1) the development of the technology to grow primary human T cells with IL-2; (2) the development of sensitive and specific assays for RT as a surrogate marker for a retrovirus; (3) providing credence in the scientific community that humans could and indeed were infected by retroviruses; (4) providing insights and the idea that AIDS might be caused by a new retrovirus; (5) providing the experience for developing a serological assay for retroviral infection based on the detection of specific anti-retroviral antibodies; (6) not well known, was our early experience in the development of inhibitors of RT (Gallo *et al.*, 1972; Schrecker *et al.*, 1972, 1974; Ting *et al.*, 1972; Yang *et al.*, 1972;), and demonstrating that such inhibitors could prevent *in vitro* infection, and more importantly prevent transmission of virus and prevention of disease in animal models (Ting *et al.* 1973, 1975) which provided a framework for drug development against HIV; and (7) showing that regulatory genes (and proteins) might be part of the strategy for viral replication in contrast to prior work with animal retroviruses, the genomes of which only harbored genes coding for structural proteins.

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